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ORIGINAL ARTICLE *Gynaecology*

Estrogen receptor β upregulates CCL2 via NF-κB signaling in endometriotic stromal cells and recruits macrophages to promote the pathogenesis of endometriosis

Yanling Gou[†], Xue Li[†], Peiling Li, Hongyan Zhang, Tenghan Xu, Hao Wang, Beidi Wang, Xuesong Ma, Xue Jiang, and Zongfeng Zhang*

Department of Obstetrics and Gynecology, Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China

*Correspondence address. Department of Obstetrics and Gynecology, Second Affiliated Hospital of Harbin Medical University, 148 Baojian Road, Harbin 150086, China. E-mail: viaac1973@163.com

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STUDY QUESTION: How is the activation of estrogen receptor β (ERβ) in endometriotic stromal cells (ESCs) involved in macrophage recruitment to promote the pathogenesis of endometriosis?

SUMMARY ANSWER: ERβ modulates the production of C-C motif chemokine ligand 2 (CCL2) via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in ESCs and thus recruits macrophages to ectopic lesions to promote pathogenesis.

WHAT IS KNOWN ALREADY: Macrophages are mainly recruited to the peritoneal cavity to promote the pathogenesis of endometriosis. Recent studies have demonstrated that ERβ plays an important role in the progression of endometriosis through modulating apoptosis and inflammation.

STUDY DESIGN, SIZE, DURATION: An observational study consisting of 22 cases (women with endometriosis, diagnosed by laparoscopy and histological analysis) and 14 controls (without endometriosis) was carried out.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Tissues and stromal cells that were isolated from 22 patients with ovarian endometrioma and deeply infiltrating endometriosis were compared with tissues and stromal cells from 14 patients with normal cycling endometrium using immunohistochemistry, quantitative PCR, Western blot, cell migration assay and cloning formation assay. P values < 0.05 were considered significant, and experiments were repeated in at least three different cell preparations.

MAIN RESULTS AND THE ROLE OF CHANCE: We observed that accumulated macrophages were recruited to the ectopic milieu and mainly adopted an alternatively activated macrophage (M2) phenotype. To reveal the underlying mechanism for this, we conducted a series of experiments and found that high expression of ERβ led to the production of CCL2 via NF-κB signaling and macrophages were recruited to the ectopic milieu. An *in vitro* co-culture assay also suggested that the recruited macrophages in turn could promote the proliferation and clonogenic ability of ESCs. Overall, the activation of ERβ in ESCs is involved in macrophage recruitment via NF-κB/CCL2 signaling and subsequently appears to promote the pathogenesis of endometriosis.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Due to the limitations of obtaining surgical specimens, endometrioma tissues were collected mainly from women diagnosed with middle to late stage endometriosis. We identified the predominant presence of M2 macrophages in the samples used in our study, but the underlying mechanism of how recruited macrophages acquire the M2 phenotype is undefined.

[†]The authors consider that the first two authors should be regarded as joint First Authors.

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WIDER IMPLICATIONS OF THE FINDINGS: This work provides novel insight into the mechanism by which ERβ may modulate macrophage infiltration and promote pathogenesis, which may provide a new therapeutic target for endometriosis.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the National Natural Science Foundation of China (81671430). The authors have no conflicts of interest.

Key words: estrogen receptor beta / macrophage / CCL2 / endometriotic stromal cell / endometriosis

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Introduction

Endometriosis (EMs) is a common chronic disease that affects ~6–10% of women of reproductive age (Simoens *et al.*, 2007). The main pathological process of EMs is characterized by the abnormal growth of vascularized endometrial tissue at ectopic sites, typically including pelvis, and causes pain, dyspareunia and infertility (Giudice, 2010). Although EMs is defined as benign, the disease not only brings a burden to society and the economy but can also significantly decrease patient quality of life. The most commonly accepted theory for EMs is the Sampson's theory, which suggests that endometrial cells and fragments reflux and implant into the pelvic cavity through the fallopian tubes during the menstrual period. However, retrograde menstruation is observed in most women, but only 10–24% of those women develop EMs (Halme *et al.*, 1984), suggesting that other factors, such as a dysregulated immune microenvironment, may play a role in the initiation and development of EMs.

The aberrant infiltration and dysregulation of immune cells and their mediators in the microenvironment are mainly responsible for the poor clearance of ectopic endometrial debris (Capobianco and Roverequerini, 2013; Li *et al.*, 2014; Yang *et al.*, 2017; Zhou *et al.*, 2017). Among these immune cells, macrophages were found to play a crucial role in EMs lesion establishment. Moreover, peritoneal macrophages from EMs patients possess a distinct reduced phagocytic capacity (Symons *et al.*, 2018). Macrophages differentiate into classically activated macrophages (M1) or alternatively activated macrophages (M2) in response to different environmental stimuli. M1 macrophages are characterized by their ability to kill micro-organisms and having a pro-inflammatory function, while M2 macrophages often exhibit immunosuppressive properties due to their abnormally secreted chemokines and decreased phagocytic ability. Currently, M2 macrophages can be further divided into four subtypes (M2a, M2b, M2c and M2d) depending on their stimulants. Among these, M2a and M2b macrophages play an important role in the immune response suppression and tissue repair during inflammation, while M2c macrophages are related to phagocytosis. M2d macrophages mainly promote tumor progression and tumor angiogenesis. In recent years, the definition of macrophage polarization has been extended and more subsets have been elucidated, such as hemorrhage-associated macrophages, macrophages stimulated with oxidized phospholipids, M4 and tumor-associated macrophages. Based on the available data regarding the characteristics of these macrophages, they regulate the immune microenviroment in a multifunctional way, in which a large number of factors can be involved (Cheng *et al.*, 2018). The polarization of M1/M2 macrophages in EMs remains highly debated in many articles. According to one study by Bacci, more M2 phenotype macrophages are recruited to the peritoneal cavity of EMs patients than to the peritoneal cavity of control patients with uterine leiomyomas (Bacci *et al.*, 2009). Meanwhile, Itoh *et al.* (2013) report that the recruitment of peritoneal M2 macrophages displays no difference between women with EMs and other benign gynecologic conditions. Studies on the cellular and molecular pathways of how macrophages are recruited to the ectopic milieu and which phenotype they mainly exhibit in the process are critical for developing potential treatments or prevention.

Studies have reported that ectopic endometriotic lesions contain higher levels of 17β-estradiol than those found in normal endometrial tissues (Delvoux *et al.*, 2009) and that the increased level of estradiol can bind and activate estrogen-dependent signaling pathways in endometriotic tissues to stimulate the corresponding biological effects. There are two classical estrogen receptors (ERs), ERα and ERβ, which are encoded by the ESR1 and ESR2 genes, respectively. ERβ was recognized to be more highly expressed in ectopic endometriotic tissues than ERα and was found to drive the progression of EMs (Han *et al.*, 2015). A detailed study by O'Malley and co-workers elucidated that enhanced ERβ expression in endometriotic tissues contributed to the pathogenesis of EMs by regulating apoptosis complexes and the inflammasome (Han *et al.*, 2015). Another study reported that a specific ERβ antagonist can inhibit estrogen-associated inflammation and affect the progression of EMs in mice (Zhao *et al.*, 2015). These two studies focus mainly on the role of ERβ in the regulation of signaling pathways within endometriotic stromal cells (ESCs). However, whether ERβ can modulate immune microenvironment components, such as macrophages, to promote the pathogenesis of EMs remains to be elucidated.

In this study, we tested for the first time the hypothesis that ERβ upregulates C-C motif chemokine ligand 2 (CCL2) via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in ESCs and thus recruits macrophages to promote the pathogenesis of EMs. The findings demonstrate that the immunoregulation role of ERβ can potentially be used as a therapeutic target for EMs.

Materials and Methods

Patients and tissue samples

This study was approved by the Ethical Committee of the Second Affiliated Hospital of Harbin Medical University. The study recruited 22 women with EMs who were diagnosed by laparoscopy and histological analysis at the Second Affiliated Hospital of Harbin Medical University from March 2017 to April 2018. Patients could be confirmed pathologically by the presence of ectopic endometrial glands and stromal cells. We collected sample tissues during surgery and immediately sent them to a laboratory. All of the tissues were divided into two samples. We conducted *in vitro* primary cell culture on one sample, and the remaining

sample was fixed with formalin and prepared for paraffin embedding processing. For the controls, ESCs were collected from 12 patients who underwent hysterectomy with other benign gynecologic conditions and without clinical indication or history of adenomyosis or EMs. All of the samples were collected during the proliferative phase of the menstrual cycle, confirmed histologically according to the established criteria and transported to the laboratory on ice in DMEM (Dulbecco's modified Eagle's medium) (Biological Industries, Israel) to isolate and culture ESCs for use in *in vitro* assays. Detailed patient information is presented in Table I. All tissue samples were obtained with the informed consent of the patients.

Primary ESCs culture

The isolation and culture of primary ectopic endometrial cells and normal endometrial cells were conducted as previously reported (Yang *et al.*, 2017). Ectopic lesions from patients with ovarian endometrioma and deeply infiltrating endometriosis were collected and minced into 1 mm pieces. The minced tissues were incubated with 4% collagenase type IV (C5138, Sigma, USA) for 40–60 min at 37°C with constant agitation. Then, the tissue pieces were filtered through 100 and 400 stainless steel mesh sieves to remove debris and isolate ESCs from epithelial cells. The filtrate was then centrifuged at 1200 *g* for 5 min and washed with DMEM three times to remove the erythrocytes. After centrifugation, we discarded the supernatant and re-suspended the remaining cells in DMEM containing 10% fetal bovine serum (FBS; Biological Industries, Israel) and plated on culture flasks. After incubation in a humidified incubator with 5% CO_2 at 37°C for 12 h, the culture medium was replaced. When the cell intensity reached 80%, trypsin-EDTA (0.25%, 25200056, Gibco, USA) was used to detach cells and conduct cell passage. The ESCs were verified through positive immunohistochemical staining of vimentin and CD10 and negative staining of E-cadherin, cytokeratin 7 (CK7) and FSH receptor (FSHR). Cells isolated from each individual patient were used for one experiment at a time in triplicate.

Immunohistochemical staining

After staining with haematoxylin and eosin to confirm the pathologic diagnosis, antigen was retrieved, and the slides were blocked with 3% hydrogen peroxide and 5% bovine serum albumin. The tissue sections were incubated overnight at 4°C with primary antibody diluted with PBS with Tween 20, and a secondary antibody kit (CWBIO, Beijing, China) was utilized to link the primary antibody. A diaminobenzidine (DAB) staining kit (CWBIO, Beijing, China) was used to develop positive staining. Details of the primary antibodies used are shown in Table II. For vimentin, CK7, E-cadherin and CD206, antigen retrieval was performed by steaming in 1 mM EDTA for 30 min. For E-cadherin, FSHR, CD68 and CD16, slides were placed in 0.25 mM Tris base buffer. Negative controls were conducted without using primary antibody.

Cell migration assay

Macrophage migration was assessed using a Corning Transwell plate (3422, Corning, NY, USA). Briefly, THP-1 (1×10^6) cells (acute monocytic leukemia cell line) were washed three times with PBS and then re-suspended in 200 µl of DMEM and added to the top of 6.5 mm-diameter, 8 µm-pore polycarbonate Transwell inserts that were subsequently placed over a 24-well plate. The lower chamber was seeded with ESCs (1×10^5), which were cultured in DMEM without serum. After 24 h, we collected the medium in the lower chamber and assessed the migrated macrophage number using Countstar. For the experiment reported in Fig. 3, 5 µM ERB-041 (524684-52-4, Sigma-Aldrich) or 10 µM PHTPP (a selective estrogen receptor beta antagonist, 805239-56-9, Sigma-Aldrich) was added to the co-culture system. For the experiment reported in Fig. 4, 500 ng/ml CCL2 (R&D Systems) was added to lentivirus transfected ERβ knockdown (sh-ERβ) ESCs, or 10 µM RS102895 (MCE, Shanghai, China) was added to ERB-041 in DMEM complete medium for 24 h to determine the migrated monocytes. Dimethylsulfoxide (DMSO) added to the co-culture system was used as a vehicle treatment.

Immunofluorescence staining

The frozen tissue sections were washed three times in PBS to wash off the OCT frozen medium. We blocked non-specific binding with 5% goat serum (CWBIO, Beijing, China), and the sections were incubated overnight at 4°C with the primary antibodies CD68 (1:50, Proteintech, Rosemont, IL, USA) and vimentin (1:50, Proteintech, Rosemont, IL, USA), washed with PBS three times, and incubated at room temperature with anti-rabbit Alexa488 (SA00006-2, Proteintech, Rosemont, IL, USA) and anti-mouse Alexa594 (SA00006-3, Proteintech, Rosemont, IL, USA) secondary antibodies for 1 h. After the sections were washed, the cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA).

Stromal cells co-cultured with macrophages

The macrophage differentiation of THP-1 cells (acute monocytic leukemia cell line), which were purchased from ScienCell, was conducted in accordance with a previously reported method (An *et al.*, 2017). The monocytes were pre-stimulated by adding 200 ng/ml phorbol 12-myristate 13-acetate

Table I Clinical characteristics of women with and without (control) endometriosis.

	Non-endometriosis group (*n* = 12)	Endometriosis group (*n* = 22)	*P* value
Age (years)	42.25 ± 1.14	40.14 ± 1.00	0.195
BMI (kg/m^2)	23.25 ± 1.23	22.68 ± 0.82	0.693
Menstrual average cycle (days)	27.45 ± 4.57	27.72 ± 3.69	0.581
Menstrual duration (days)	6.04 ± 1.58	6.44 ± 1.46	0.484
ASRM stage			
Stage 1		0/22	
Stage 2		3/22	
Stage 3		7/22	
Stage 4		12/22	

*Significant difference at $P < 0.05$. Statistical analysis was performed using a two-tailed Student's *t*-test. Data are mean ± SD. ASRM, American Society for Reproductive Medicine.

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Table II Details of antigens used in immunohistochemistry analyses of human endometriotic stromal cells.

Antigen	Catalog number	Dilution	Source	Species
Vimentin	60330-1-Ig	1:200	Proteintech	Mouse mAb
Cytokeratin 7	66483-1-Ig	1:500	Proteintech	Mouse mAb
E-cadherin	60335-1-Ig	1:2000	Proteintech	Mouse mAb
FSHR	22665-1-AP	1:200	Proteintech	Rabbit
CD68	25747-1-AP	1:100	Proteintech	Rabbit
CD16	16559-1-AP	1:200	Proteintech	Rabbit
CD206	18704-1-AP	1:200	Proteintech	Rabbit
ESR2	14007-1-AP	1:50	Proteintech	Rabbit

ESR, estrogen receptor; FSHR, FSH receptor; mAb, monoclonal antibody.

(PMA) (Sigma-Aldrich, St. Louis, MO, USA) in complete culture medium for 2–4 days. When most monocytes were attached to the plate and appeared to be stretching out, the stimulation was suspended by replacing culture medium back to non-PMA-containing medium.

Cell sorting by flow cytometry

The co-cultured macrophages were digested and washed with cold PBS three times and then incubated with primary antibodies and the corresponding secondary antibodies at 4°C. The results were assessed using BD FACSDIVA software (BD Biosciences, CA, USA).

Plasmid construction and transfection

The sh-ERβ plasmid was constructed in the LV4 lentiviral vector as reported previously (Rao *et al.*, 2016). After packaging in 293 T cells, lentivirus supernatant was collected and frozen at −80°C for later use in the transfection of stromal cells. The lentivirus supernatant was added to complete culture medium at a multiplicity of infection of 100 along with 5 μg/ml polybrene. After transfection for 48–72 h, green fluorescent protein expression was observed under a fluorescence microscope to evaluate the transfection efficiency. The efficiency of ERβ knockdown in cells was evaluated by Western blotting (Supplementary Fig. S1).

Western blotting analysis

Whole cell and nuclear protein extraction was performed as described previously (Itoh *et al.*, 2017). After protein was transferred to polyvinylidene fluoride (PVDF) (Millipore, Billerica, MA, USA) membranes, the membranes were blocked with 5% non-fat milk and blotted with ERβ (14007-1-AP, Proteintech), p65 (ab6503, Abcam), IκBα (protein inhibitor of NF-κB transcription factor, 10268-1-AP, Proteintech) and GAPDH (HRP-60004, Proteintech) antibodies for 1 h at 37°C. After washing with PBS with 0.1% Tween, membranes were incubated for 2 h at room temperature with secondary antibodies. Quantification of protein expression with respect to GAPDH was conducted with ImageJ software (National Institutes of Health, USA).

Total RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. A quantitative real-time PCR (qRT-PCR) assay was conducted as previously described (Rao *et al.*, 2016). The primer pair sequences were ^{5}AGCACGGCTCCATATACATACC^{3} (sense) and ^{5}TGGACCACTAAAGGAGAAAGGT^{3} (antisense) (to amplify ERβ), ^{5}CAGCCAGATGCAATCAATGCC^{3} (sense) and ^{5}TGGAATCCTGAACCCACTTCT^{3} (antisense) (to amplify CCL2), and ^{5}GGAGTCAACGGATTTGGTCGTA^{3} (sense) and ^{5}CAACAATATCCACTTTACCAGAGTTA^{3} (antisense) (to amplify GAPDH). The Bio-Rad CFX96 system was used with SYBR green (TOYOBO, Shanghai, China) to determine the mRNA expression levels. Expression levels were normalized to the expression of GAPDH RNA. The mean relative gene expression was determined, and differences were calculated using the $2^{-\triangle\triangle Ct}$ method (Han *et al.*, 2015).

Cytokine antibody array

The cytokine profiles of the supernatant samples from ESCs were determined using RayBio® Cytokine Antibody Array G-Series 3 containing 58 cytokines (AAH-CYT-G3-8, RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Supernatant was collected from ESC samples with or without ERβ knockdown. The intensities of cytokines on the films were quantified using ImageQuant TL (GE Healthcare, Buckinghamshire, UK). The intensity of each spot was normalized according to positive controls.

Measurement of CCL2 and CCL8 levels

Conditioned media (CM) was collected from ESCs with lentivirus-transfected plasmid for ERβ and negative control or from different treatment groups for 48 h. CM was used for the detection of CCL2 and CCL8 using human CCL2 (KE00091, Proteintech, Wuhan, China) and CCL8 (RAB0079, Sigma-Aldrich, St. Louis, USA) ELISA kits according to the manufacturer's instructions. Concentration (pg/ml) was normalized to total cell protein.

Chromatin immunoprecipitation assays

Ectopic ESCs were pre-treated with PHTPP (10^{-5} M), sh-ERβ, ERB-041 (10^{-5} M), ERB-041 plus ammonium pyrrolidinedithiocarbamate (PDTC, a NF-κB activation inhibitor, 10^{-4} M) or vehicle for 24 h. Cells were fixed and subjected to chromatin immunoprecipitation (ChIP) analyses as previously described (Nozell *et al.*, 2008; Xing *et al.*, 2012). Extracted DNA was purified using mini spin columns, and immunoprecipitated and non-immunoprecipitated DNA (Input) were analyzed by real-time PCR using the following specific primers: IκBα forward, ^{5}CTCCGAGACTTTCGAGGAAATAC^{3} and reverse, ^{5}GCCATTGTAGTTGGTAGCCTTCA^{3} for the human IκBα gene promoter; CCL2 forward ^{5}CAGCCAGATGCAATCAATGCC^{3} and reverse, ^{5}TGGAATCCTGAACCCACTTCT^{3} for the human CCL2 gene promoter. The results are representative of three experiments.

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Animal studies

A mouse model of EMs was established as described (Zhang *et al.*, 2018). Ten-week-old C57BL/6 female mice were purchased from the Laboratory Animal Service Center of the Second Affiliated Hospital of Harbin Medical University and housed in the animal lab. All animal experiments were approved by the Institutional Animal Care and Use Committee. Briefly, we removed uterine horns from the donor mice and added them to DMEM medium. Endometrium was divided into 1 mm^2 fragments. Endometrial fragments from each uterine horn were suspended in 0.3 ml sterile PBS and injected into the peritoneal cavities of recipient mice with an 18-gauge needle. The mice were sacrificed on Days 3, 5 and 7, and endometriotic lesions were collected. The length and width of the lesions were measured, and the volume of lesions was calculated by the prolate ellipsoid geometric model: $(\text{length} \times \text{width}^2)/2$ (Naito *et al.*, 1986).

Bromodeoxyuridine cell proliferation

The isolated ectopic ESCs from women with EMs were treated with CCR2 antagonist (RS102895, at 10 μM) for 24 h. In addition, DMSO was added as a negative control. Then, the ability of ESCs to proliferate was detected by bromodeoxyuridine cell proliferation assay kits (Millipore, USA) according to the manufacturer's instructions.

In vitro serial cloning

Individual large stromal colony forming units (CFUs) from passage 1 (P1) were trypsinized using cloning rings (Sigma-Aldrich) to determine the self-renewal capacity of the cells and co-cultured with autologous macrophages. Three individual large CFUs per patient sample ($n = 3$) obtained from the clonogenic assays were used. The cell number of each CFU was determined, and the cells were re-seeded at a density of 20 cells/cm^2. This process continued until the cells could no longer form CFUs.

Statistical analysis

Unless otherwise indicated, we performed experiments three times and collected the data. Data are shown as the mean ± SD. All statistical analyses were performed using SPSS 16.0 software (IBM, USA), and graphical representations were performed with GraphPad Prism 5 (San Diego, CA, USA) software. Student's *t*-test or one-way ANOVA were used to analyze the differences between means. A P value < 0.05 was considered to be statistically significant. In all figures, relevant comparisons were labeled as either significant at the $P \leq 0.001$ (***), $P \leq 0.01$ (**), or $P \leq 0.05$ (*) level, or non-significant (ns) for values of $P > 0.05$.

Results

EMs tissues recruit more macrophages than normal endometrial tissues

Early studies reported that macrophages could be recruited to various chronic inflammation tissues (Moore *et al.*, 2013). To explore whether EMs, as a chronic inflammatory disease, has an increased capacity to recruit macrophages, we performed immunohistochemical staining of CD68, a common marker for macrophages, in human EMs samples for comparison to normal endometrial tissues. We found that macrophage infiltration was significantly greater in EMs than in normal endometrial tissues (Fig. 1A and B). To confirm these results *in vitro*, we used a Transwell chamber migration system (Fig. 1C) to assay the ability of THP-1 cells to migrate towards ectopic and normal ESCs. The results showed that ectopic ESCs recruited more macrophages than normal ESCs (Fig. 1D and E). Taken together, results using human tissue samples and *in vitro* cell migration assays showed that EMs tissues could recruit more macrophages than normal endometrial tissues.

Macrophages in EMs lesions mainly present the M2 phenotype

To further identify the polarization statues of recruited macrophages, we applied immunohistochemical staining of CD16 and CD206 in human EMs tissues, and the results revealed that the recruited macrophages in EMs lesions mainly present the M2 phenotype but not the M1 phenotype during the formation of endometriotic lesions. Consistent results were also found in lesion samples obtained from the mouse EMs model (Fig. 2A and B). In addition, M2 macrophages can be found mostly near the stromal cells, which are indicated by the immunofluorescence staining of vimentin (Fig. 2C). To validate the results *in vitro*, we co-cultured THP-1-derived macrophages with ectopic and normal ESCs. Flow cytometry showed that CD206 expression was significantly higher after co-culturing with ESCs (Fig. 2D and E), indicating that the recruited macrophages polarize to the M2 phenotype under the influence of cytokines from stromal cells. Collectively, these data suggested that M2 macrophages were mainly present in endometriotic lesions.

ERβ modulates macrophage infiltration both *in vitro* and *in vivo*

It has been reported that ERβ is highly expressed and associated with the inhibition of apoptosis in EMs (Han *et al.*, 2015). Consistent with this, we also found that ERβ was expressed more highly in human and mouse ectopic endometriotic tissues compared to normal endometrial tissues (Fig. 3A). The elevated expression of ERβ was also confirmed at the mRNA and protein levels (Fig. 3B and C). An *in vitro* migration assay showed that migrated THP-1 cells were significantly decreased in ERβ-knockdown supernatants compared to supernatants of the negative control (Fig. 3D). To confirm the role of ERβ *in vivo*, we injected i.p. PHTPP, a selective ERβ antagonist, into immunocompetent mice and collected the EMs lesions. Immunostaining of CD68 in paraffin-embedded tissues suggested that PHTPP-treated lesions failed to recruit macrophages (Fig. 3E). The above results suggest ERβ modulates macrophage infiltration in endometriotic lesions.

ERβ modulates CCL2 production to promote macrophage infiltration in EMs

To explore the mechanism by which ERβ mediates macrophage infiltration, we performed a macrophage-associated cytokine protein array. The results revealed decreased CCL2 and CCL8 expression in CM after the knockdown of ERβ in ESCs (Fig. 4A). ELISA confirmed that the knockdown of ERβ in ESCs remarkably inhibited CCL2 but not CCL8 expression in ESCs (Fig. 4B). The expression of *CCL2* was also confirmed at the mRNA level (Fig. 4C). To verify whether ERβ promotes macrophage infiltration via the CCL2/CCR2 pathway, we added recombinant CCL2 or CCR2 antagonists to the migration assay system, in which ERβ is selectively activated or inhibited in ESCs. As

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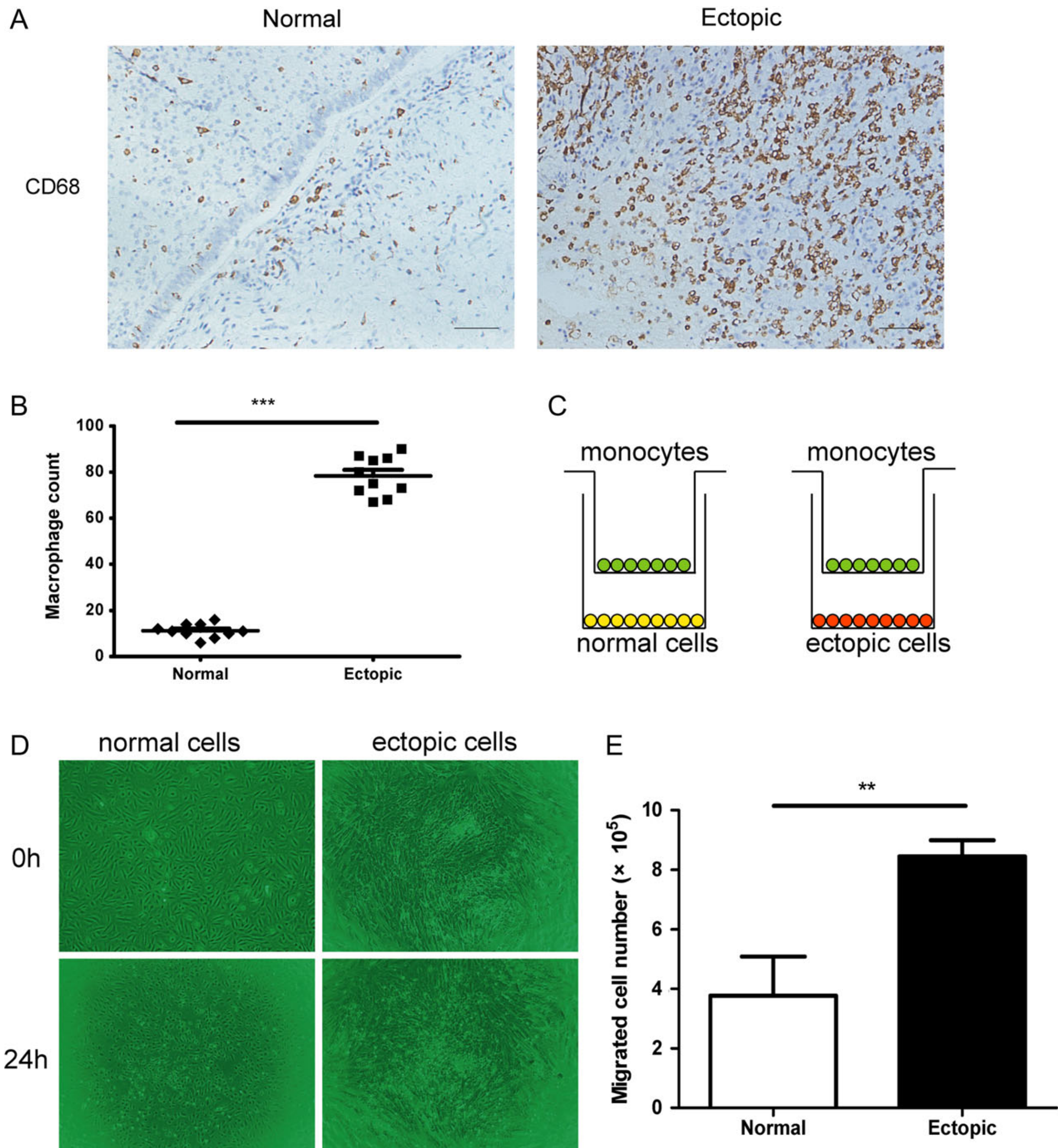

Figure 1 Endometriosis tissues recruit more macrophages than normal endometrial tissues. (**A**) Immunohistochemical staining of CD68 as a cell marker to detect macrophages in human endometriosis (EMs) and normal endometrial tissues (macrophages are stained dark brown, original magnification ×200). (**B**) Quantification of macrophage counts in EMs (n = 10) and normal endometrial tissues (n = 10) (mean ± SD of numbers of macrophages per five fields of view at ×200 magnification). (**C**) Cartoon illustration of the macrophage migration assay. The upper insert chambers with a 4 μm pore polycarbonate membrane were pre-coated with 10 ng/ml fibronectin. THP-1 cells, an acute monocytic leukemia cell line (1 × 10E6), were placed in the insert, and ectopic endometriotic or normal endometrial stromal cells were added into the bottom wells to assay the migration rate of monocytes. (**D**) After a 12 h incubation of monocytes and stromal cells, the bottom sides of the insert wells were captured, and the numbers of migrated monocytes were quantified using a Countstar automated cell counter. (**E**) Quantitation data for migrated monocytes. The results are presented as the mean ± SD. Statistical analysis was performed using a two-tailed Student's t-test. ***$P \le 0.001$, **$P \le 0.01$.

expected, adding recombinant CCL2 can abrogate the reduction of THP-1 cell migration induced by ERβ knockdown in ESCs. The presence of CCR2 antagonist can block the accumulation of macrophages mediated by ERβ activation (Fig. 4D). The above results showed that ERβ-mediated macrophage infiltration in EMs was due to increased production of CCL2.

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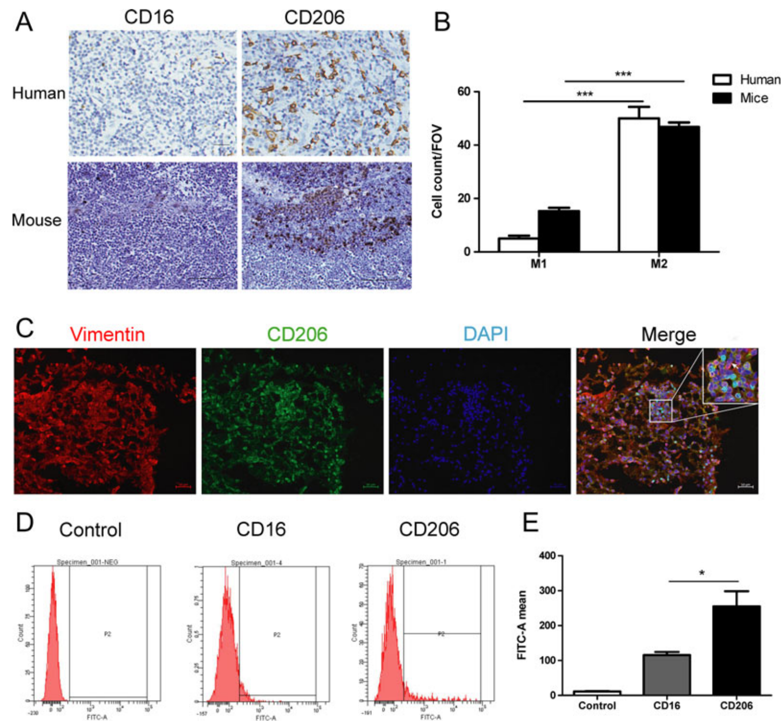

Figure 2 Macrophages in EMs lesions mainly present the M2 phenotype. (**A**) Immunohistochemical staining of CD16 (M1) and CD206 (M2) to detect the distribution of macrophage subsets in human endometriotic tissues (upper panel) and established mouse endometriosis ectopic lesions (lower panel). (**B**) Quantification of the subsets of macrophages in human and mouse EMs endometriosis ectopic lesions (mean ± SD of numbers of macrophages per five fields of view at ×200 magnification). One-way ANOVA was used to analyze the differences between means. (**C**) Demonstrative immunofluorescence staining of macrophages (black arrow) surrounding endometriotic stromal cells (white arrow). Stromal cells are indicated by vimentin (red), and macrophages are stained by CD206 (green) and 4′-6-diamidino-2-phenylindole. (**D**) Representative plots by flow cytometry analysis in THP-1-derived macrophages co-cultured with endometriotic stromal cells. (**E**) Comparison of the FITC-means between CD16 and CD206 in co-cultured macrophages. Experiments were repeated three times. Statistical analysis was performed using a two-tailed Student's *t*-test. $^{***}P \leq 0.001$, $^{*}P \leq 0.05$.

ERβ upregulates CCL2 expression through NF-κB signaling

ERβ is a transcription factor that targets a large number of signaling molecules. We reviewed the results of genome-wide mapping of ERβ binding regions in intact chromatin from the cultured breast cancer cell line MCF-7 (Grober, 2011). Among the primary ERβ target genes identified from next-generation sequencing technologies combining ChIP, we chose to investigate NF-κB signaling, which mediates the early inflammatory response. To investigate the impact of ERβ on NF-κB signaling, we extracted the total protein and nuclear proteins from ERβ knockdown ESCs and assessed the levels of total IκBα and nuclear p65 using Western blot. The results revealed that the total levels of IκBα were significantly elevated while p65 decreased (Fig. 5A). Next, ChIP assays were performed, and we found that the levels of ERβ at the IκBα promoter were significantly increased and that p65 at the CCL2 promoter was decreased in the presence of PHTPP or ERβ knockdown in ESCs, while PDTC, a NF-κB antagonist, significantly reversed the level of ERβ at the IκBα promoter increased by ERβ knockdown (Fig. 5B and C). Concerning the regulation of CCL2 production, we collected supernatants from ESCs under different treatments and determined the levels of cytokine CCL2 using ELISA. The results showed that NF-κB antagonist significantly suppressed CCL2 levels and abrogated CCL2 production increased by ERβ agonist (Fig. 5D). Taken together, these results suggest that ERβ upregulates CCL2 expression through NF-κB signaling.

Aberrant recruitment of macrophages in endometriotic lesions promotes the progression of EMs

To further corroborate the role of recruited macrophages and their contribution to the establishment of endometriotic lesions, we injected CCR2 antagonists i.p. into recipient mice. Blocking CCL2 signaling by CCR2 antagonist significantly suppressed both the size and weight of endometrial lesions. There was a dramatic inhibition of the development of endometrial lesions at Day 7 after endometrial implant transfer (Fig. 6A and B). To investigate the role of macrophages on stromal cells *in vitro*, we co-cultured M2 macrophages and ESCs and calculated the proliferation rate of stromal cells compared with non-macrophage ESC culture as a control. The results showed that co-culture with M2 macrophages significantly promoted the proliferation of ESCs (Fig. 6C). In addition, an *in vitro* serial cloning assay revealed that co-cultured ESCs exhibited increased ability to form CFUs (Fig. 6D). These data suggested that the

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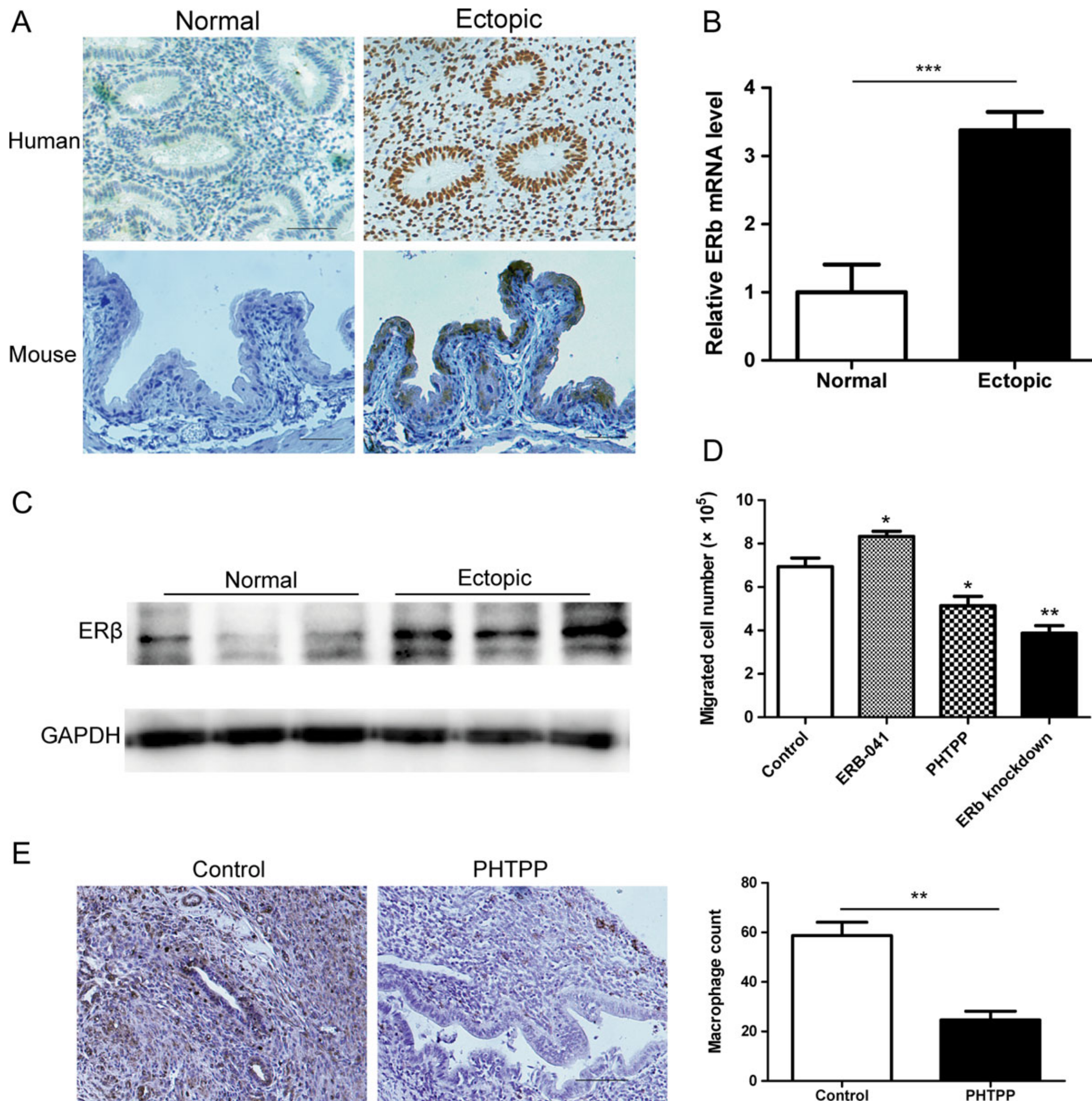

Figure 3 **Estrogen receptor β modulates macrophage infiltration both *in vitro*** and *in vivo*. (**A**) Immunostaining of estrogen receptor beta (ERβ) in human and mouse normal endometrial tissues and ectopic endometriotic tissues. (**B**) Quantitative RT-PCR (qRT-PCR) was used to determine the relative levels of ERβ mRNA expression normalized to the housekeeping gene GAPDH. The normal group was normalized to 1. (**C**) ERβ protein expression in endometriotic stromal cells and normal endometrial stromal cells was determined by Western blot. (**D**) Migration assay of primed THP-1 cells towards endometriotic stromal cells with recombinant C-C motif chemokine ligand 2 (CCL2) or ERβ antagonist (PHTPP) or lentivirus transfected ERβ knockdown. Endometriotic stromal cells transfected with the negative control was compared as a control. $^{*}P < 0.05$ compared with control; $^{**}P < 0.01$ compared with control. (**E**) Immunostaining of CD68 in endometriotic lesions in two EMs mouse model groups following treatment with dimethylsulfoxide (DMSO) or PHTPP. The numbers of infiltrated macrophages were counted based on an image of view at 200× magnification. Statistical analysis was performed using a two-tailed Student's *t*-test. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$.

aberrant recruitment of macrophages to endometriotic lesions promoted the progression of EMs.

Discussion

In this study, we found compelling evidence, both *in vivo* and *in vitro*, that ERβ promotes the pathogenesis of endometriotic lesions by modulating macrophage infiltration. We observed accumulated macrophages recruited to the ectopic milieu that mainly adopted the M2 phenotype. To reveal the underlying mechanism, we conducted a series of experiments and found that highly expressed ERβ can lead to the production of CCL2 via NF-κB signaling and thus recruit macrophages to the ectopic milieu. An *in vitro* co-culture assay also suggested that the recruited macrophages in turn can promote the proliferation of ESCs and enhance their clonogenic ability.

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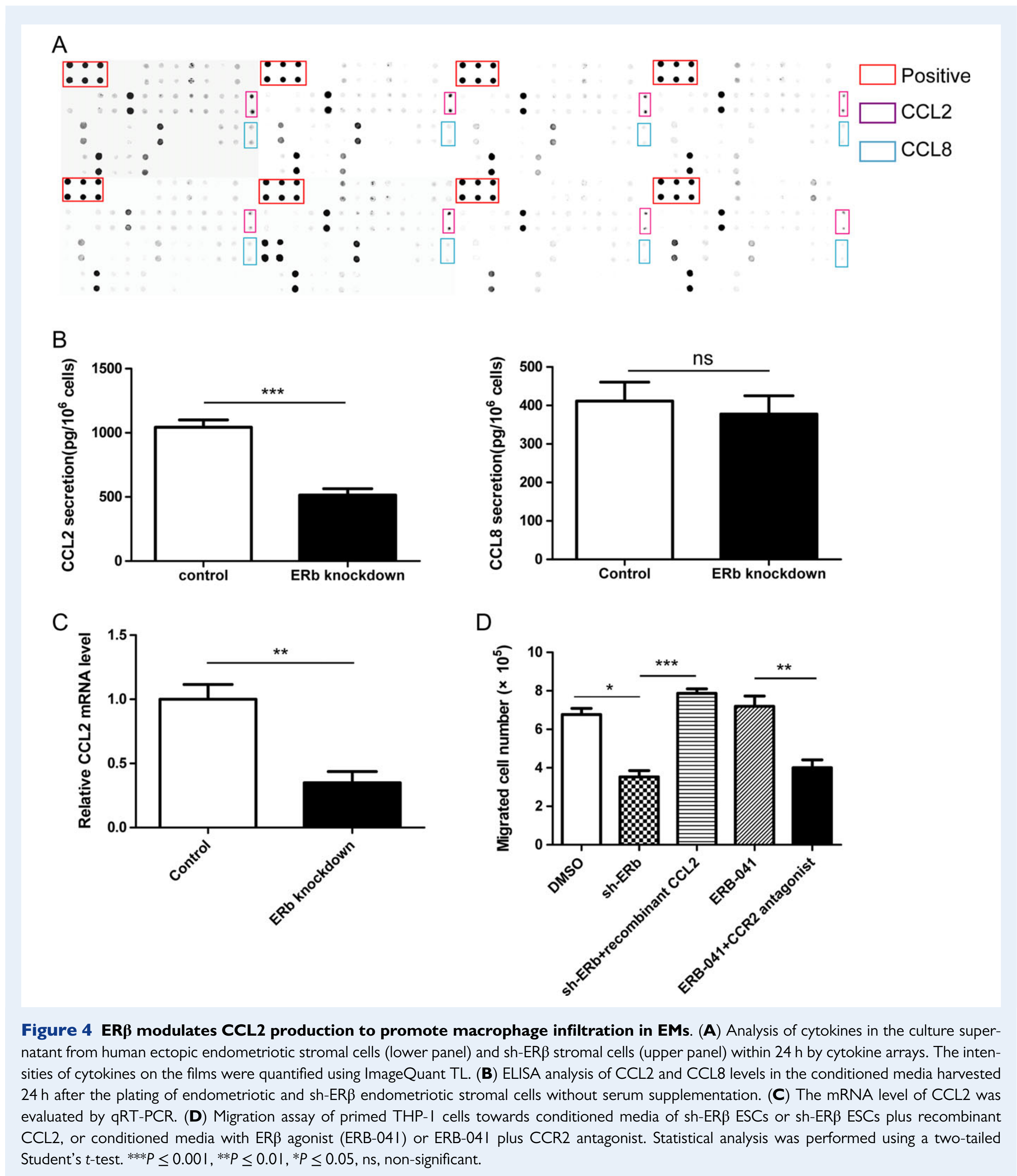

Figure 4 ERβ modulates CCL2 production to promote macrophage infiltration in EMs. (**A**) Analysis of cytokines in the culture supernatant from human ectopic endometriotic stromal cells (lower panel) and sh-ERβ stromal cells (upper panel) within 24 h by cytokine arrays. The intensities of cytokines on the films were quantified using ImageQuant TL. (**B**) ELISA analysis of CCL2 and CCL8 levels in the conditioned media harvested 24 h after the plating of endometriotic and sh-ERβ endometriotic stromal cells without serum supplementation. (**C**) The mRNA level of CCL2 was evaluated by qRT-PCR. (**D**) Migration assay of primed THP-1 cells towards conditioned media of sh-ERβ ESCs or sh-ERβ ESCs plus recombinant CCL2, or conditioned media with ERβ agonist (ERB-041) or ERB-041 plus CCR2 antagonist. Statistical analysis was performed using a two-tailed Student's *t*-test. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, ns, non-significant.

Recent studies demonstrate that susceptibility to EMs is the consequence of poor clearance of refluxed endometrial cells by the host immune response and enhanced endometrial cell adhesion to the peritoneum (Berkkanoglu and Arici, 2003), which is a complicated process that requires a large subset of dysregulated immune cells, including macrophages, neutrophils, myeloid-derived suppressor cells, dendritic cells, natural killer cells and B cells. Our study discovered markedly more infiltrated macrophages in the ectopic milieu, which is consistent

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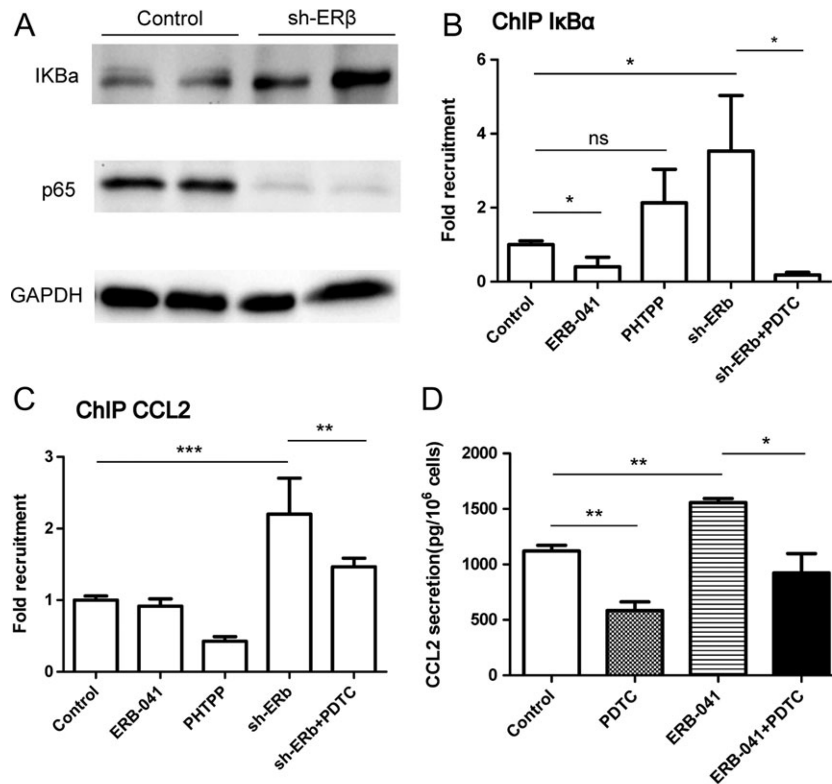

Figure 5 **ERβ upregulates CCL2 expression through nuclear factor kappa-light-chain-enhancer of activated B cell signaling**. (**A**) The protein expression of IκBα and p65 in sh-ERβ ESCs was analyzed by Western blot. (**B** and **C**) ChIP samples were prepared as described in the text and analyzed using antibodies specific for ERβ and p65. The immunoprecipitated DNA fragments and input DNA were analyzed by real-time PCR. The numbers represent the mean ± SD from three experiments repeated in duplicate. (**D**) ELISA analysis of CCL2 levels in the conditioned media harvested 24 hours after plating endometriotic stromal cells with DMSO, PDTC (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) antagonist), ERB-041 or ERB-041 plus PDTC. Statistical analysis was performed using a two-tailed Student's *t*-test. ***$P \le 0.001$, **$P \le 0.01$, *$P \le 0.05$, ns, non-significant.

with a previous study that demonstrated that macrophages were increased in the peritoneal cavity and exhibited impairment of their phagocytic activities (Chuang *et al.*, 2009; Capobianco and Roverequerini, 2013). In addition, we also found that macrophages largely resided near the ESCs, indicating that macrophages may be recruited by cytokines secreted by ESCs. These results suggest that macrophages may play an important role in the pathogenesis of EMs and may serve as a potential therapeutic target.

Recently, ERβ has emerged as an important player in the pathogenesis of EMs. Studies have found that cells in human endometriotic lesions, either ovarian or deeply infiltrating endometriosis, display higher ERβ expression when compared to that of normal human endometrial cells (Bulun *et al.*, 2012). The ERβ to ERα ratio was over 1 in both human and animal models of EMs lesions, while ERα was predominately expressed in normal endometrial cells (Fazleabas *et al.*, 2003; Greaves *et al.*, 2014; Králíčková and Vetvicka, 2015). Recent studies have demonstrated that ERβ plays an important role in the progression of EMs by modulating apoptosis and inflammation (Han *et al.*, 2015; Zhao *et al.*, 2015). However, little is known about the role of ERβ in the immune microenvironment of EMs lesions. In this study, we uncovered that macrophages were recruited to endometriotic foci in conditions of ERβ activation both *in vitro* and *in vivo*. Collectively, our study defines a novel role for ERβ in regulation of the immune microenvironment in EMs.

Previous studies have shown that some chemokines are produced by stromal cells and play a significant role in the infiltration of immune cells into the peritoneal cavity (Hornung *et al.*, 2001; Zhang *et al.*, 2018). This process was thought to mediate the inflammatory microenvironment in EMs. CCL2 has been identified as one of the key chemokines that regulate the migration and infiltration of macrophages in cancers (Liu *et al.*, 2017). To identify the mechanism of ERβ involvement in macrophage infiltration in endometriotic lesions, we studied the mutual communication between ESCs from ectopic lesions and macrophages at a cytokine level in a simulative ectopic microenvironment *in vitro*. The results revealed that ERβ acted as a gene to promote CCL2 production in EMs. We also demonstrated that ERβ can activate NF-κB pathways to achieve this effect. In the ChIP assay, adding NF-κB antagonist only partially reversed the effect of ERB-041, indicating that there may be other pathways to mediate the function of ERβ in CCL2 regulation. Thus, the results demonstrated that inhibitors targeting the ERβ/NF-κB/CCL2 axis may be useful for application in clinical EMs treatment.

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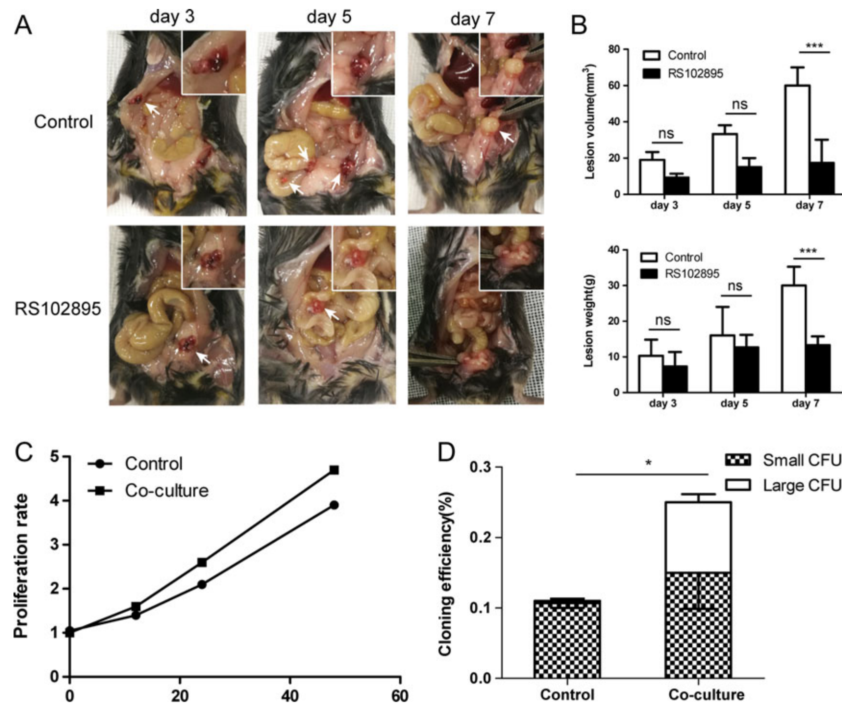

Figure 6 **Aberrant recruitment of macrophages in endometriotic lesions promotes the progression of EMs**. (**A**) Development of lesions (black arrows) at Day 3, Day 5 and Day 7 in mice or mice injected with CCR2 antagonist. (**B**) Comparison of the size and weight of endometrial lesions in the control group ($n = 12$) and that in the RS102895 group ($n = 12$). All bar graphs show the mean ± SD. One-way ANOVA was used to analyze the differences between means. (**C**) The effect of M2 macrophage co-culture on the proliferation rate of ectopic ESCs. (**D**) Cloning efficiency (CE) of stromal cells after co-culture with M2 macrophages for 10 days. Statistical analysis was performed using a two-tailed Student's *t*-test. $^{***}P \leq 0.001$, $^{*}P \leq 0.05$, ns, non-significant.

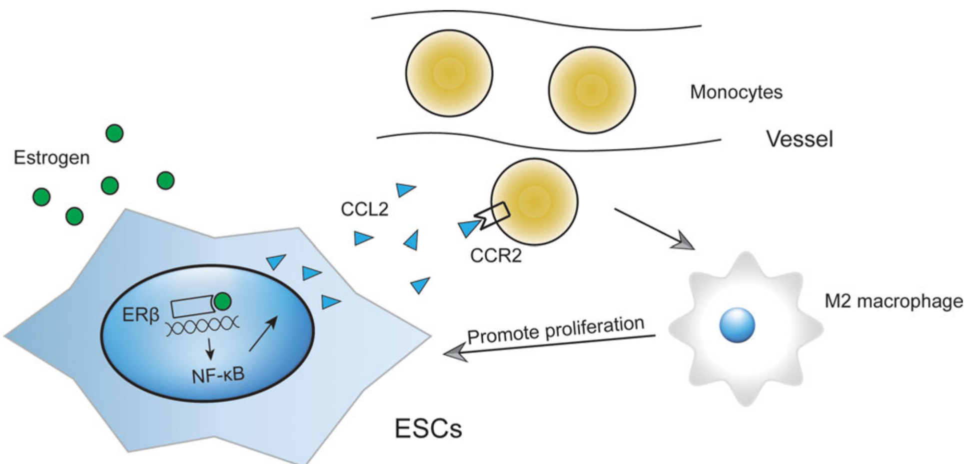

Figure 7 **Cartoon illustration of ERβ activating NF-κB/CCL2 signaling in endometriotic stromal cells and recruiting macrophages to promote the pathogenesis of EMs**.

The ectopic localization of endometrial tissue fragments requires a series of immune responses, including the recruitment of macrophages, blood vessels, cytokines and nerve fibers into the resultant lesions. In our study, we discovered that the cell count number of M2 macrophages is higher than that of M1 macrophages in human endometriotic lesions and that undifferentiated macrophages are also significantly polarized to the M2 phenotype after co-culture with ectopic ESCs *in vitro*. Previous studies implied that M2 macrophages might

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promote migration, invasion, and tissue repair in EMs (Wang *et al.*, 2014; Woo *et al.*, 2017; Duan *et al.*, 2018). In this study, our *in vitro* data indicated that the co-culture of M2 macrophages with ESCs can promote the proliferation and clonogenic ability of ESCs and contribute to the process of EMs formation, which further indicated that crosstalk between macrophages and EMs exists and may contribute to the development of EMs.

M1 macrophages are activated by microbial components such as lipopolysaccharide and/or cytokines such as interferon-γ or granulocyte-macrophage colony-stimulating factor, while different cytokines such as interleukin (IL)-4, IL-13, IL-10 or macrophage colony-stimulating factor can lead to the activation of M2 macrophages. Based on the fact that M2 phenotype macrophages were present in greater numbers than M1 phenotype macrophages in the microenvironment of ectopic EMs, we speculated that some cytokines that can alternatively activate macrophages could exist. However, after analyzing the data from the cytokine antibody array of ERβ knockdown supernatant, cytokine that alternatively activates macrophages was not found. This result indicated that ERβ may not be involved in M2 phenotype differentiation, and other molecular pathways might be responsible for this process. The mechanism by which recruited macrophages polarize to the M2 phenotype is undefined, and we plan to focus on this aspect in our following research.

In conclusion, our study demonstrates that ERβ modulates the production of CCL2 via NF-κB signaling in ESCs and thus recruits macrophages to ectopic lesions to promote the pathogenesis of EMs (Fig. 7). The ERβ/CCL2/macrophage axis may be a potential therapeutic target for EMs treatment.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

Y.G. and X.L. participated in study design, execution, acquisition of data, analysis. P.L., H.Z. and T.X. were responsible for critical discussion and experiment execution. H.W. and B.W. participated in writing and revising article. X.M. and X.J. conducted experiment execution and identification of patients. Z.Z. participated in study design, execution, analysis, critical discussion and final approval of the version to be published.

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Conflict of interest

The authors have no conflicts of interest.

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